REMARKS

I. Description of the Present Invention

The present invention relates to a two-part method for the detection of nucleic acid target sequences, wherein a detection procedure may be employed in combination with an amplification procedure to achieve the detection of low levels of target in a test sample. Amplification is accomplished by using a denatured amplification sequence of the target as a template for the contiguous hybridization of a plurality of denatured pairs of complementary amplification probes. Ligation of the contiguously hybridized probes generates amplification product which can then be denatured from the amplification sequence, thereby releasing additional template (denatured amplification product) to guide the contiguous hybridization of amplification probes in subsequent cycles of the amplification reaction. This enables the exponential accumulation of amplification product.

Where three or more pairs of amplification probes are employed, appropriately designed detection probes can be used to distinguish correctly assembled amplification product from incorrectly assembled spurious amplification by-product. This type of spurious by-product is formed in solution without benefit of template sequence and, therefore, is not indicative of the presence of target in the test sample. If the presence of the spurious by-product is not minimized or distinguished in some way from the desired amplification product, the by-product creates serious background problems, negatively impacting sensitivity.

At least two detection probes are used in the present method, with each detection probe being complementary to a portion of each of two adjacently situated amplification probe segments of the amplification product. The correctly assembled amplification product serves as a template for the contiguous hybridization of the detection probes to form a detection product. The detection product can then

be joined to form a ligated detection product in a manner similar to the ligation of contiguously hybridized amplification probes during amplification. Where the detection probes are joined, the resulting ligated detection product can, for example, be separated by polyacrylamide gel electrophoresis (PAGE).

Claims 1-5 are directed to the amplification procedure of the present invention, with claims 6-13 covering the detection procedure. The combined amplification/detection procedure is covered in claims 14-18. Claims 19 and 20 are directed to reagents for the amplification and detection procedures, respectively, and claim 21 is directed to a kit, or set of reagents, for use in the combined amplification/detection procedure.

II. 35 USC §103 Objections

The Examiner has rejected all of claims 1-21 of the present application under 35 USC §103 as being unpatentable over Carr and Whiteley *et al* in view of Mullis *et al.* Mullis *et al* teach an amplification method whereby the target sequence is used as a template for the formation of a complementary strand through primer extension using a polymerase. The complementary strand thus formed is denatured and utilized as template in subsequent cycles of amplification to achieve geometric accumulation of copies of a duplex target. On the other hand, Carr and Whiteley *et al* teach assay methods using a nucleic acid template for the hybridization of two contiguous probes which are subsequently ligated, with the ligated product then being denatured. According to the Carr and Whiteley *et al* references, a complementary strand to a nucleic acid template can be formed from ligating fragments together either with a ligase (Carr and Whiteley *et al*) or by use of a polymerase and ligase (Carr). According to the Examiner:

> One of ordinary skill in the art would know [from Mullis *et al*] that what was important was the formation of the complementary sequence and that whether one used

short fragments (amplification probes) or DNA and a polymerase or short fragments of DNA and a ligase with or without a polymerase that the only thing of importance was the formation of a complementary strand which could be used in subsequent reactions.

There is no teaching or suggestion in the prior art which would lead one to combine Mullis with Carr and/or Whiteley *et al.* The latter two references are directed to analytical methods which do not contemplate amplification. (Carr seeks to eliminate background from nonspecific hybridization of detection probes to carrier DNA in a target sample, while Whiteley *et al* disclose lengthening a labeled probe used for the detection of an immobilized target sequence for a similar purpose, i.e., to ameliorate the background problem presented by nonspecific and/or partial hybridization of the labeled detection probe to non-target nucleic acid.) Both the Carr and Whiteley *et al* methods take advantage of the higher T_m of the longer ligated probes to selectively denature the shorter unligated probes (which are not hybridized to target sequence), thereby removing these background-causing spuriously bound probes from the reaction mixture.

Moreover, mere combination of the cited references does not in any way suggest Applicants' "combined" amplification/detection system which successfully discriminates against spurious blunt-end ligated amplification by-product. The background caused by target-independent ligation of non-hybridized probes can defeat the entire purpose of the amplification procedure by masking results from samples at the sensitivities which require target amplification in the first place. The occurrence of spurious by-product is particularly problematic, because the spuriously formed products serve as template in subsequent cycles to produce the exponential formation of amplification product in the absence of target sequence, thus limiting the sensitivity of the method. The background created by this type of by-product is such a serious problem in ligase-based

types of amplification procedures that it can be self-defeating to the amplification method.

It is significant that others have recognized the severity of the blunt-end ligation problem in ligase-based amplification systems. European Patent Application No. 320,308 ("Backman *et al*"), for example, discloses a similar type of ligase-based amplification system, but provide only limited suggestions directed to reduction of the amount of spurious ligation product formed, namely by: (1) phosphorylating only the abutting ends of the probes; (2) using probes of unequal length; and, (3) limiting the number of amplification cycles. The first two suggestions are limited to the preferred four probe Backman *et al* amplification system, inasmuch as only the terminal probes are available for this type of manipulation. (The Backman *et al* approach to the problem of spurious blunt-end ligated amplification by-product is not effective, however, in Applicants' preferred amplification procedure using three or more pairs of amplification probes because the middle pair(s) of probes cannot be manipulated in the manner taught by Backman *et al.*) The last suggestion limits the sensitivity of the amplification procedure, yielding a result which is self-defeating.

It is even more significant that the blunt-end ligation problem continues to be recognized by others with respect to ligase-based amplification systems. For example, the problem was recently reported by Barringer *et al*, *Gene*, *89*, 117-122 (1990), a copy of which is attached hereto as Exhibit A, wherein it is suggested that the amount of undesired target-independent ligation can be minimized by reducing the amounts of the two reagents which participate in blunt-end ligation, namely the amplification probes and the *E. coli* ligase. Reducing the amounts of these reagents, however, negatively impacts the kinetics of the amplification reaction. Even more recently, Kwoh *et al*, *Am. Biotech. Lab.*, *8*(13) 14-25 (1990), a copy of which is attached hereto as Exhibit B, also report that the ligases used in ligase-based amplification schemes are prone to yielding blunt-end ligated by-products, because of the large

excess of free nucleic acid probes required to drive the amplification reaction forward.

In contrast to the suggestions of others in the field, who continue to chase the elusive goal of reducing or minimizing the amount of blunt-end by-product formed, Applicants' method allows the blunt-end by product to form (thereby avoiding unnecessary negative implications on the reaction efficiency) and instead discriminates the non-target-derived blunt-end ligated product from the desired amplification product. With Applicants' unique detection system, incorrectly aligned spurious blunt-end ligated amplification by-product cannot act as a template for hybridization of the detection probes. As a result, the detection product serves as an indication of only the correctly assembled amplification product, nearly all of which is traceable to the presence of target.

This unique approach enables Applicants to make use of increasing the number of pairs of probes to improve sensitivity in an assay, because the small percentage of correctly assembled by-product (which is not discriminated from the desired template-derived amplification product) decreases even further with increasing numbers of pairs of amplification probes, as set forth in the specification. In contrast, increasing the number of pairs of amplification probes is detrimental to a system which must rely on reducing or minimizing the formation of the background-causing by-product. The ability to discriminate the undesired blunt-end ligation by-product enables much greater sensitivity to be achieved by Applicants' method than is possible with the latter type of system.

Applicants have amended independent claims 14 (method) and 21 (diagnostic kit) to require a minimum of at least three pairs amplification probes and at least two detection probes

III. 35 USC §112 Objections

The Examiner has rejected claims 19-21 under 35 USC §112, first paragraph, on the grounds that the disclosure is enabling only for claims limited to a plurality of denatured pairs of amplification probes. Claims 1 and 14 have previously been amended to reflect that a plurality of denatured pairs of amplification probes participates in the amplification of the target sequence. It is, however, possible for pairs of amplification probes to be added in hybridized form to the amplification reaction mixture of the present invention. It is a simple matter, well known to those skilled in the art, to denature the hybridized pairs of probes subsequent to their addition to the reaction mixture, but prior to the amplification reaction, in order to enable the resulting denatured pairs of amplification probes to hybridize to the target sequence. Providing the amplification probe reagent as hybridized pairs of amplification probes has the advantage of insuring that the pairs of probes are stoichiometrically "paired", as taught in the present specification. For this reason, claims 19-21 have not been amended to include the limitation that the plurality of pairs of amplification probes be provided in denatured form. Where amplification probe reagents comprising hybridized pairs of amplification probes are provided, a denaturing step can quickly and easily be performed prior to commencement of the amplification procedure.

The Examiner has also rejected claims 2, 3, 10, 11, 16, and 17 under 35 USC §112, first paragraph, on the basis of the disclosure being limited to a thermostable ligase. The Examiner has suggested that the scope of these claims should be narrowed. In fact, the use of a thermostable ligase is preferred, as taught in the specification, because it eliminates the need for the addition of fresh reagent with each cycle, as is required for ligases which are not thermostable. The examples in the present specification, however, demonstrate the use of both T4 DNA ligase and *E. coli* ligase, neither of which is thermostable. Applicants have, however, amended to incorporate the limitation that a ligase (claims 2, 10, and 16) and a thermostable

ligase (claims 3, 11, and 17) be employed to join the contiguously ligated probes.

Claim 19 has been rejected under 35 USC §112, first and second paragraphs, for lack of enablement and/or for failing to particularly point out and distinctly claim the subject matter of the invention. Specifically, the Examiner states that the term "sufficiently adjacent", which appears in the claim in question, fails to particularly point out and distinctly claim the invention, because the specification is enabling for a one nucleotide gap only where ligation is concerned, because no fill-in reactions are disclosed. Applicants have amended claim 19 to incorporate the language suggested by the Examiner, namely: --Amplification probes binding to template sequence (Amplification sequence) in a contiguous manner having a gap of no more than one nucleotide between said amplification probes--.

The Examiner has rejected claims 1-21 under 35 USC §112, first paragraph, because "the disclosure is enabling only for claims limited to a target nucleic acid sequence wherein the nucleotide sequence is known." Applicants contend that the identity of the nucleic acid sequence need not be known in all instances. For example, it may only be necessary to know the amino acid sequence of the final protein product encoded by a particular nucleic acid sequence in order to make effective use of the present invention. In this case, it is possible to use oligonucleotide probe mixtures, based on the protein sequence of the resulting gene product and the degeneracy of the DNA code, to identify and characterize the nucleic acid sequences responsible for encoding these gene products. Applicants wish to point out, however, that it is not critical that the probes be 100% homologous, as noted by the Examiner. It is only critical that the probes have sufficient complementarity to enable hybridization to occur. In some instances, it may be beneficial to employ probes having less than complete complementarity with the target. (See copending U.S. Patent Application No. 517,631.)

The Examiner has further rejected claims 1-21 under 35 USC §112, first and second paragraphs, for lack of enablement and/or for failing to particularly point out and distinctly claim the subject matter of the invention. The Examiner states that functional language should be recited in the claims regarding probe length, as the limitations of the specification must be read into the claims. However, the Examiner notes that "the specification fails to teach probe length, and is therefore not enabling for a plurality of denatured pairs of amplification probes. Because probe number and respective lengths are critical to the hybridization reactions, as it is both probe length and excess concentration of probe that will 'drive the reaction forward'."

While both probe length and excess concentration of probe contribute to the kinetics which drive the reaction forward, it is known to those skilled in the art that an excess of probes beyond 10^8, as will typically be required to effect a ligase-based amplification system, the kinetics of the hybridization reaction becomes linear with respect to probe length. Therefore, probe length is not critical with respect to reaction kinetics in this instance. Moreover, the actual probe length in a particular assay can readily be ascertained by one of ordinary skill in the art in light of the teachings of the present invention. The specification teaches that the probes should be at least two oligonucleotides long. Examples are provided wherein probe lengths of 15 nucleotides are employed. The preferred lengths will, of course, be dictated in part by well known teachings in the hybridization assay art which prescribe that the more complex the carrier DNA in a sample (e.g., a target with human gene carrier DNA), the longer the probe (or, in this case, the sum of the amplification probes) must be to distinguish the target over the carrier DNA. (See, for example, Helene *et al*, *Control of Gene Expression by Oligodeoxynucleotides Covalently Linked to Intercalating Agents and Nucleic Acid-Cleaving Reagents*, in Oligodeoxynucleotides -- Antisense Inhibitors of Gene Expression, CRC Press, Inc., 139-142 (Cohen, ed., 1989), a copy of which is attached hereto as Exhibit C, wherein various means of calculating

the optimum size(s) required for an oligonucleotide probe to recognize a single specific sequence in a genome are discussed.) In the case of Applicants' amplification method, it is the overall length of the contiguously hybridized amplification probes must distinguish the target sequence from, e.g., carrier DNA.

Claims 6, 7, 9, 14, and 21 are rejected under 35 USC §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicants regard as the invention. In this regard, claims 6, 7, 9, 14, and 21 are viewed as being vague and indefinite in their "recitation of 'a different combination of amplification probe segments'." In dependent claims 6, 14 and 21, Applicants have amended the claim language to the language as originally filed, i.e., the previously added limitation "a different combination of" has been deleted from the claim. It is believed that the previously added language is unnecessary in light of the requirement that the detection probes hybridize in a contiguous manner.

For the foregoing reasons, Applicants believe the present application is in condition for allowance, and an early notification to that effect is earnestly solicited.

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